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Mutations Affecting Lipopolysaccharide Enhance Ail-Mediated Entry of *Yersinia enterocolitica* into Mammalian Cells

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Two genes of *Yersinia enterocolitica*, *inv* and *ail*, have been identified as having a role in the bacterial adherence to and entry into mammalian cells in vitro. Expression of both genes is regulated by temperature. In stationary phase, *ail* gene expression is detectable only in bacteria at 37°C, not at lower temperatures. An *inv* mutant derivative of *Y. enterocolitica*, which cannot enter mammalian cells when grown at 30°C because of the lack of both *inv* and *ail* gene products, was mutagenized with the transposons mini-Tn*10* and Tn*5B50* to look for an increase in Ail-mediated cell entry. Sixteen mutants that could enter tissue culture cells after growth at 30°C were selected. All of the mutants had increased cell surface Ail levels as detected by an Ail-specific monoclonal antibody. All of the ten Tn*5B50* and one of the six mini-Tn*10* mutants showed no increase in *ail* expression, but they had alterations in their lipopolysaccharide (LPS) such that no O side chains were detectable in bacteria grown at 30°C. Thus, these mutants that are increased in their ability to enter cells appear to be so as a result of a change in the LPS on the surface resulting in increased levels of Ail protein able to interact with the mammalian cell surface. In the remaining mini-Tn*10* mutants, LPS is normal, and the increase in cell surface Ail levels appears to be due to an increase in *ail* mRNA present in the cell. These mutants may therefore be affecting a repressor of *ail* gene expression.

The interaction between pathogenic bacteria and mammalian cells in vitro has been used as a model system for studying bacterium-host cell interactions during the normal course of infection. The *inv* and *ail* genes of *Yersinia enterocolitica*, a gastrointestinal pathogen, were identified as having a role in bacterium-host cell interactions by using one of these in vitro models. When either gene is introduced into normally noninvasive laboratory strains of *Escherichia coli*, the resulting recombinants are able to adhere to and enter cultured mammalian cells (14). *E. coli* cells containing the *ail* gene adhere to a wide variety of tissue culture cell types. However, entry is cell type specific; *E. coli* cells containing the *ail* gene enter only Chinese hamster ovary (CHO) cells to any measurable degree (14). An *ail* mutant derivative of *Y. enterocolitica* is impaired in both adherence to and entry into CHO cells (19). Reintroduction of a wild-type copy of *ail* into this mutant restores the ability to adhere and enter mammalian cells.

The *ail* gene product has also been implicated in bacterial resistance to complement-mediated killing. Laboratory strains of *E. coli* containing the *ail* gene and nonpathogenic *Y. enterocolitica* strains containing the *ail* gene on a recombinant plasmid are over 10,000 times more resistant to killing by human serum than are their parental strains lacking the *ail* gene (4, 19). *ail* mutant derivatives of a pathogenic *Y. enterocolitica* strain are highly sensitive to serum killing; this serum-sensitive phenotype is complemented by a wild-type copy of the *ail* gene (4, 19).

In addition to Ail, the O-antigen side chain of lipopolysaccharide (LPS) of *Y. enterocolitica* has been shown to be important for resistance to serum killing. Activation of the alternative complement pathway is inhibited by LPS in *Y. enterocolitica* serotype O:3 strains (28). The O side chain is also important in a number of other members of the family *Enterobacteriaceae* for resistance to complement killing. Selection for increased serum resistance in clinical *E. coli* isolates results in cells with more highly substituted O side chains (20). O polysaccharide purified from serum-resistant *E. coli* and *Salmonella typhimurium* isolates can protect serum-sensitive strains from killing by serum (25). In the case of *Salmonella montevideo*, the O-antigen side chain protects the cell from being killed by sterically hindering access of complement components to the bacterial cell surface (10).

The expression of a number of *Y. enterocolitica* genes, including the *ail* gene and the genes required for synthesis of O polysaccharide of the O:3 type, the *rfb* genes, are regulated by temperature and growth phase (1, 19). The *ail* gene is expressed in cells growing logarithmically at 30 and 37°C but not at lower temperatures. In stationary-phase cells, however, *ail* transcripts are detectable only at 37°C (19). This lack of expression of the *ail* gene in stationary-phase cells at 30°C could be due to the presence of a repressor in these cells under these conditions or due to the absence of a transcriptional activator. Here I describe the isolation of mini-Tn*10* and Tn*5B50* mutants to begin to identify potential regulators of *ail* gene expression.

MATERIALS AND METHODS

Bacterial strains and tissue culture cells. The *E. coli* and *Y. enterocolitica* strains used in this study are described in Table 1. Bacteria were grown in LB broth (Gibco) from single colonies on LB plates. *E. coli* strains were grown at 37°C and *Y. enterocolitica* strains were grown at 30°C unless otherwise indicated. Conjugation mixtures were plated on minimal A plates (12) to select against the multiply auxotrophic *E. coli* donor. The antibiotics kanamycin and chloramphenicol (each at a concentration of 50 μg/ml; Sigma Chemical Co.) and tetracycline (15 μg/ml; Sigma) were added to the media where appropriate. The mini-Tn*10* transposon conferring resistance to tetracycline, referred to as element 8 by Way et al. (29), was used for mutagenesis. This transposable element contains the outer 78 bp of IS*10*-Right flanking the tetracycline resistance

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
Y. enterocolitica		
8081c	*inv*$^+$ *ail*$^+$ invasive	21
DP5102	Cm^r *inv* derivative of DP8014	This study
DP5142	Km^r derivative of DP5102	This study
DP5143	Km^r *ail* derivative of DP5102	This study
DP8014	Cm^r derivative of 8081c	This study
DP5102::mini-Tn*10* 2c5	Mini-Tn*10* derivative of DP5102	This study
DP5102::mini-Tn*10* 1-1	Mini-Tn*10* derivative of DP5102	This study
DP5102::mini-Tn*10* 1-2	Mini-Tn*10* derivative of DP5102	This study
DP5102::mini-Tn*10* 3-2	Mini-Tn*10* derivative of DP5102	This study
DP5102::mini-Tn*10* 4-1	Mini-Tn*10* derivative of DP5102	This study
DP5102::mini-Tn*10* 5-1	Mini-Tn*10* derivative of DP5102	This study
DP5102::mini-Tn*10* 2c5-52	Km^r *ail* derivative of DP5102::mini-Tn*10* 2c5	This study
DP5102::mini-Tn*10* 2c5-53	Km^r derivative of DP5102::mini-Tn*10* 2c5	This study
DP5102::Tn*5B50* 1-1	Tn*5B50* derivative of DP5102	This study
DP5102::Tn*5B50* 1-2	Tn*5B50* derivative of DP5102	This study
DP5102::Tn*5B50* 2-1	Tn*5B50* derivative of DP5102	This study
DP5102::Tn*5B50* 2-2	Tn*5B50* derivative of DP5102	This study
DP5102::Tn*5B50* 2-3	Tn*5B50* derivative of DP5102	This study
DP5102::Tn*5B50* 3-1	Tn*5B50* derivative of DP5102	This study
DP5102::Tn*5B50* 3-2	Tn*5B50* derivative of DP5102	This study
DP5102::Tn*5B50* 4-1	Tn*5B50* derivative of DP5102	This study
DP5102::Tn*5B50* 4-2	Tn*5B50* derivative of DP5102	This study
DP5102::Tn*5B50* 4-3	Tn*5B50* derivative of DP5102	This study
DP5102 2c5-13	Tc^r derivative of DP5102	This study
E. coli		
HB101	*supE hsdS recA ara proA lacY galK rpsL xyl mtl*	5
SM10(λpir)	*thi thr leu tonA lacY supE pir* RK6 *recA*::RP4-2-Tc::Mu Km	15
Plasmids		
pJM703.1	Ap^r, R6K ori, RP4 oriT	15
pACYC184	Cm^r, Tc^r, P15A	6
pDP111	5.7-kbp *inv* + flanking sequences in pACYC184	This study
pDP14	5.7-kbp *inv* + flanking sequences in pJM703.1	This study
pDP1422	1.7-kbp Cm^r gene in *inv* in pDP14	This study
pFSVΔailkm	1.5-kbp Km^r gene in 1.5-kbp *ail* in pFSVΔNR	This study
pNK861	6.1-kbp mini-Tn*10* + transposase in pBR333	29
pDP149	6.1-kbp mini-Tn*10* + transposase from pNK861 in pJM703.1	This study
pJM703.1::Tn*5B50*	pJM703.1 + Tn*5B50*	D. Schauer
pMT11HC	Ap^r, ColE1	18
pACYC184-km	Km^r, Tc^r, P15A	This study
p227	10.8-kb *Sau*3A partial of 8081c chromosomal DNA in pACYC184	This study
p227-km	Km^r derivative of p227	This study

gene from Tn*10*. The transposase gene, expressed from the *tac* promoter, is located outside the transposon ends. Insertions of this transposon are stable because the element does not encode transposase in its new genetic environment. Tn*5B50* (23) is a tetracycline-resistant derivative of Tn*5* containing a full-length IS*50*-Right with a functional transposase gene. Only the outer 53 bp of IS*50*-Left are present on the other side of the tetracycline resistance gene. Between the IS*50*-Left sequences and the tetracycline resistance gene is an outwardly oriented *nptII* promoter. Insertions of this transposon can not only inactivate genes, as would occur following insertion of any transposon, but also activate gene expression from the *nptII* promoter if the insertion occurs upstream of a coding sequence. CHO cells (ATCC CCL61) were grown at 37°C in 5% CO_2 atmosphere in minimal essential medium supplemented with nonessential amino acids, glutamine, and 5% fetal bovine serum (Gibco). HEp-2 cells (ATCC CCL 23) were grown in RPMI 1640 supplemented with glutamine and 5% fetal bovine serum.

DNA and RNA isolation and manipulations. Restriction enzyme digestions and ligations were performed as described by Maniatis et al. (11). Chromosomal DNA isolated as described previously (22) was digested with *Eco*RI or *Ava*I, subjected to electrophoresis, and transferred to nitrocellulose. RNA was isolated and subjected to electrophoresis as described previously (19). A mini-Tn*10*-specific probe was prepared by gel elution of the two mini-Tn*10*-specific *Eco*RI fragments of pNK861 (29). An internal 3.5-kbp *Hin*dIII Tn*5B50* fragment (23) isolated by gel elution was used to probe for Tn*5B50*-specific hybridization. The *ail*-specific probe was a 0.4-kbp *Cla*I-*Acc*I fragment of pVM103 containing sequences within the *ail* open reading frame (13). These probes were hybridized to filters by standard techniques (11, 19).

Construction of *inv* and *ail* mutants and an *inv ail* double mutant. The *inv* mutant DP5102 was constructed by gene replacement in the following manner. The *Bam*HI fragment of pVM111 (16) containing the *inv* gene was introduced into the *Cla*I site in plasmid pJM703.1 (15) after addition of *Xba*I linkers to both molecules giving compatible ends for ligation, yielding pDP14. A 1.7-kbp *Bcl*I fragment from pDPT270 (24) containing a gene for chloramphenicol acetyltransferase was inserted into the *Cla*I site within the *inv* protein-coding se-

quence of pDP14, creating pDP1422. *E. coli* SM10(λpir) containing pDP1422 was mated (12) with *Y. enterocolitica* 8081c, and chloramphenicol-resistant transconjugants were selected on minimal A plates. One transconjugant, DP8014, was subcultured daily in LB broth without chloramphenicol for 8 days, after which individual colonies were screened for loss of pJM703.1-homologous sequences by colony hybridization (11). Chromosomal DNA was analyzed from segregants that had lost plasmid sequences to identify those isolates that had retained the mutated *inv* allele. To check for complementation of the *inv* mutation, the 5.7-kbp *Bam*HI fragment of pVM111 was introduced into the *Bcl*I site of pACYC184, forming pDP111, and this plasmid was introduced in DP5102 by electroporation (7).

An *ail* mutant derivative of DP5102 was constructed as follows. The *ail* gene was truncated in vitro by introducing the 1.4-kbp *Acc*I-*Ava*I fragment of pVM103 into the suicide plasmid pFSVΔNR (19), thereby removing the 5′ end of the gene. The gene for aminoglycoside 3′-phosphotransferase (*apt*) from pUC-4K (Pharmacia) was then introduced into the *Cla*I site in the protein-coding region of the *ail* gene. This construction is similar to that described by Pierson and Falkow (19), who used a different selectable marker. SM10(λpir)[pFSVΔailkm] was crossed with 8081c, selecting for kanamycin-resistant transconjugants on minimal A plates. Southern analysis (11) with an *ail*-specific probe (19) was used to identify whether the transconjugants were *ail* mutants, containing two mutated copies of the *ail* gene, one with the truncation and the other with the insertion (DP5143), or whether they were *ail*$^+$, retaining one wild-type copy and one doubly mutated copy of the *ail* gene (DP5142).

The same *ail* mutation was introduced into DP5102::mini-Tn*10* 2c5 by crossing this strain with SM10(λpir)[pFSVΔailkm] and selecting for kanamycin-resistant transconjugants on minimal A plates. An *ail*-specific probe (19) was used for Southern analysis (11) to determine whether the transconjugants were *ail* mutants, containing one truncated copy of the *ail* gene and one copy containing the *apt* insertion (DP5102::mini-Tn*10* 2c5-52), or whether they were wild type for *ail*, containing one wild-type copy of the *ail* gene and one doubly mutated copy (DP5102::mini-Tn*10* 2c5-53).

Isolation of mutants that suppress the inability of DP5102 to enter cells. Plasmid pNK861 containing mini-Tn*10* was subjected to partial digestion with the restriction enzyme *Eco*RI, and a 6.1-kbp fragment containing the transposon and linked transposase gene was introduced into *Eco*RI-digested pJM703.1, forming pDP149. The plasmid containing transposon Tn*5B50* was a gift of D. Schauer. SM10(λpir)[pDP149] or SM10(λpir)[pJM703.1::Tn*5B50*] was crossed with DP5102, and tetracycline-resistant transconjugants were selected on minimal A plates. Five pools of each set of transconjugants were grown to stationary phase at 30°C and added to CHO cells according to the standard cell entry assay procedure (see below). Bacteria that grew on plates after gentamicin treatment were resuspended in LB broth. Bacteria from this mixture were diluted 1:100 in LB broth containing tetracycline, grown to stationary phase at 30°C, and then added to CHO cells as before. Colonies that grew on plates after passage through CHO cells were tested individually for entry into CHO cells, and those that entered at a level similar to or higher than that of the parental strain were analyzed further.

Cell entry assays. Assays were performed as described elsewhere (18). Briefly, bacteria grown to stationary phase were added to a monolayer of tissue culture cells at a multiplicity of 100. After a 2-h incubation, nonadherent bacteria were washed off and medium containing the antibiotic gentamicin (100 μg/ml; Sigma) was added. The cells were incubated for another 2 h, after which the medium was washed away, the cells were lysed, and dilutions were plated to determine viable counts of bacteria. Efficiency of entry is expressed as number of bacteria surviving gentamicin divided by number of viable bacteria added at the start of the assay times 100. The results presented are averages of duplicate samples performed on the same day with the same bacteria and tissue culture cell samples.

Serum resistance assays. Assays of serum resistance were performed with normal human serum as described previously (19). Heat-inactivated serum was prepared by incubating the serum at 56°C for 35 min. Results from these assays are presented as $\log_{10}$ kill, which is the $\log_{10}$ CFU surviving exposure to heat-inactivated serum minus the $\log_{10}$ CFU surviving exposure to untreated human serum.

Whole cell ELISA analysis. Enzyme-linked immunosorbent assays (ELISAs) were performed with an Ail-specific monoclonal antibody (4) (a gift of J. Bliska), using equal numbers of stationary-phase bacteria as described previously (19). Briefly, equal numbers of bacteria were washed with Tris-buffered saline (TBS; 25 mM Tris, 140 mM NaCl, 3 mM KCl [pH 8.0]), diluted 10- or 100-fold, and placed into microtiter dishes. Bacteria were incubated with the Ail-specific monoclonal antibody, diluted to 50 μg/ml in TBS for 1 h on ice. After the bacteria were washed with TBS, 0.7 μg of alkaline phosphatase-conjugated anti-mouse antibody (Sigma) was added. Following a 1-h incubation on ice, the bacteria were again washed with TBS. The ELISA was developed with *p*-nitrophenyl phosphate, and the A_{410} was read on a microtiter plate reader (Flow Laboratories).

LPS gels. LPS was prepared from bacteria grown aerobically to stationary phase. Approximately 5×10^9 cells were pelleted for 1 min at 10,000 × *g* and then resuspended in 0.25 ml of H_2O. An equal volume of lysis buffer (2) was added, and the samples were boiled for 5 min. Proteinase K was added to the samples at a final concentration of 1.5 mg/ml. After a 3-h incubation, the samples were boiled again, and 20 μl each was electrophoresed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) in a 15% polyacrylamide gel containing 4 M urea. The gel was silver stained by the method of Tsai and Frasch (26).

Isolation and characterization of a clone that carries the wild-type gene mutated in DP5102::mini-Tn*10* 2c5. Chromosomal DNA from DP5102::mini-Tn*10* 2c5 digested with *Hin*dIII was ligated into *Hin*dIII-digested pMT11HC (18). This library was introduced into HB101, selecting for tetracycline resistance, the marker carried by mini-Tn*10*. Since *Hin*dIII cuts once in the transposon, the tetracycline-resistant clone isolated, called p2c5, also contained sequences on one side of the insertion. To isolate the wild-type copy of the gene, a library from 8081c was constructed by eluting *Sau*3A partially digested chromosomal DNA in the 8- to 15-kbp size range from a gel (30) and ligating this into *Bam*HI-digested pACYC184 (6). HB101 containing this library was probed (11) with the *Hin*dIII insert of p2c5. Six clones that hybridized were isolated; one of these, p227, was chosen for further analysis. A derivative of p227 resistant to kanamycin, p227-km, was constructed by introducing the *Bam*HI fragment of pUC-4K containing the *apt* gene into the *Bcl*I site within the vector portion of p227. The same *Bam*HI fragment was introduced into the *Bcl*I site in pACYC184, forming pACYC184-km, which was used as a vector control.

Deletions were introduced into p227 by digesting the plasmid DNA with restriction enzymes and replacing the deleted fragment with the *apt* gene fragment from pUC4-K. A deletion

derivative of p227 missing a 4-kbp *Cla*I fragment not needed for complementation was used for Tn*5* mutagenesis with λ::Tn*5* (8).

Construction of mutant DP5102 2c5-13. A derivative of DP5102 containing an insertion directed to the position of the mini-Tn*10* insertion in the mutant DP5102::mini-Tn*10* 2c5 was constructed as follows. The *Hin*dIII fragment from p2c5 containing a portion of mini-Tn*10* and flanking sequences was introduced into a *Hin*dIII partial digest of pJM703.1, forming pJM2c5. This plasmid was conjugated from SM10(λpir) into DP5102, selecting for tetracycline-resistant transconjugants on minimal A plates. These transconjugants should have the plasmid pJM2c5 integrated into the chromosome at the same locus as the mini-Tn*10* insertion in DP5102::mini-Tn*10* 2c5. A single transconjugant, called DP5102 2c5-13, was selected for characterization.

RESULTS

Rationale. Identification of mutants that are altered in temperature regulation of *ail* gene expression would be aided if there were a method to select for a change in one of the Ail-mediated phenotypes. Since both *inv* and *ail* act as cell entry factors when introduced into *E. coli*, it seemed likely that wild-type *Y. enterocolitica* makes use of either to enter mammalian cells. Use of increased entry as a selection for increased *ail* gene expression would therefore require that the strain be *inv* mutant. This *inv* mutant strain should enter only due to the *ail* gene product, assuming that there are no other cell entry factors in *Y. enterocolitica*. This *inv* mutant strain could then be mutagenized, and bacteria that were able to enter CHO cells better than their parent, perhaps as a result of an increase in Ail levels, could be selected by passage through CHO cells. To determine if this method would be feasible, i.e., if without Inv, *Y. enterocolitica* entry is mediated by Ail, an *inv* mutant and an *inv ail* double mutant of *Y. enterocolitica* were constructed by gene replacement methods as described in Materials and Methods.

Entry into mammalian cells by an *inv ail* double mutant. The *inv* mutant strain, DP5102, was tested for its ability to enter CHO and HEp-2 cells. As has been seen with an *inv* mutant *Y. enterocolitica* strain characterized by others (17), when the *inv* mutant strain was grown at 30°C, it was unable to enter CHO cells. However, when this strain was grown at 37°C, conditions in which *ail* is expressed, a low level of entry into CHO cells was seen (data not shown).

The *inv* mutant is able to enter CHO cells only under the conditions in which the *ail* gene is expressed. These results, and the fact that the mutant does not enter HEp-2 cells, no matter what the growth conditions (data not shown), suggest that entry by the *inv* mutant is Ail mediated. To test whether this low level of entry is indeed due to the Ail protein, an *ail* mutation was introduced into DP5102, and the resulting mutant, DP5143, was tested for entry into CHO cells when grown at 30 or 37°C (Table 2). The *inv ail* double mutant was drastically reduced in its ability to enter CHO cells when grown at 37°C compared with its parent, further supporting the hypothesis that the low level of entry by DP5102 is Ail mediated.

Isolation of mutations that suppress the inability of the *inv* mutant to enter cells. Since an increase in expression of *ail* or another, as yet unidentified cell entry factor at 30°C, either by inactivation of a putative repressor or by activation of a putative activator, should allow entry by the *inv* mutant, the transposons mini-Tn*10* (29) and Tn*5B50* (23) were introduced into the chromosome of the *inv* mutant by conjugation as described in Materials and Methods. Both transposons could be used to identify a repressor of *ail* gene expression by insertional inactivation of the repressor gene. In addition, Tn*5B50* contains the neomycin phosphotransferase gene promoter oriented near one end of transposon such that transcription proceeds outward from the transposon. Insertion of Tn*5B50* results in constitutive transcription of sequences downstream of the point of insertion. Insertion of this transposon upstream of a putative activator of *ail* transcription could thus result in an increased level of Ail protein in the cell. Pools of transposon mutants were grown to stationary phase at 30°C and then incubated with CHO cells, using the standard cell entry protocol (see Materials and Methods). Bacteria that survived exposure to gentamicin were plated after lysis of CHO cells. Colonies that grew on plates were resuspended, regrown, and repassaged through CHO cells. After the second passage, the entry into CHO cells by independent isolates grown at 30°C was examined. One of the six mini-Tn*10* mutants, DP5102::mini-Tn*10* 2c5, and all of the ten Tn*5B50* mutants entered CHO cells at levels 15- to 70-fold higher than the level of the parental strain grown at 30°C and 5- to 20-fold higher than the

TABLE 2. Effect of temperature on entry of a *Y. enterocolitica inv ail* double mutant into CHO cells

Strain	Entry efficiency[a]	
	Grown at 30°C[b]	Grown at 37°C
8081c	37.7	51.2
DP5142	0.42	2.13
DP5143	0.633	0.004
E. coli	0.067	0.132

[a] Defined as (number of bacteria surviving gentamicin treatment/number of bacteria added at the start of the assay) × 100.

[b] In all experiments, strain 8081c was grown at 30°C and *E. coli* strains were grown at 37°C. The temperatures indicated are the growth temperatures of DP5142 and DP5143 in the two experiments.

TABLE 3. Entry of DP5102 transposon mutants into CHO cells

Strain[a]	Entry efficiency[b]	
	Expt 1	Expt 2
8081c	53.2	46.0
DP5102	0.987	0.346
DP5102, 37°C		1.11
DP5102::Tn*5B50* 1-1		13.3
DP5102::Tn*5B50* 1-2		5.44
DP5102::Tn*5B50* 2-1		7.10
DP5102::Tn*5B50* 2-2		18.3
DP5102::Tn*5B50* 2-3		18.5
DP5102::Tn*5B50* 3-1		6.61
DP5102::Tn*5B50* 3-2		6.23
DP5102::Tn*5B50* 4-1		6.34
DP5102::Tn*5B50* 4-2		22.8
DP5102::Tn*5B50* 4-3		13.0
DP5102::mini-Tn*10* 2c5[pACYC184-km]	19.8	
DP5102::mini-Tn*10* 2c5[p227-km]	0.124	
DP5102::mini-Tn*10* 1-1		0.714
DP5102::mini-Tn*10* 1-2		1.03
DP5102::mini-Tn*10* 3-2		1.35
DP5102::mini-Tn*10* 4-1		1.31
DP5102::mini-Tn*10* 5-1		1.37

[a] Bacteria were grown at 30°C to stationary phase except where indicated otherwise.

[b] Determined as described for Table 2.

TABLE 4. Surface expression of Ail in the hyperinvasive mutants[a]

Strain	A_{410}/1.5 × 10^8 CFU		
	Expt 1	Expt 2	Expt 3
DP5102	0.038 ± 0.018	0.043 ± 0.042	−0.001 ± 0.031
DP5102, 37°C	1.48 ± 0.111	0.237 ± 0.067	0.109 ± 0.026
DP5102[pACYC184-km]	0.007 ± 0.022		
DP5102[p227-km]	0.021 ± 0.030		
DP5102::mini-Tn*10* 2c5[pACYC184-km]	8.02 ± 0.488		
DP5102::mini-Tn*10* 2c5[p227-km]	0.004 ± 0.024		
DP5102::Tn*5B50* 1-1		6.14 ± 1.95	
DP5102::Tn*5B50* 1-2		7.99 ± 2.64	
DP5102::Tn*5B50* 2-1		9.72 ± 4.06	
DP5102::Tn*5B50* 2-2		10.3 ± 1.26	
DP5102::Tn*5B50* 2-3		8.67 ± 0.42	
DP5102::Tn*5B50* 3-1		8.84 ± 0.78	
DP5102::Tn*5B50* 3-2		12.0 ± 3.09	
DP5102::Tn*5B50* 4-1		9.26 ± 1.14	
DP5102::Tn*5B50* 4-2		9.30 ± 0.47	
DP5102::Tn*5B50* 4-3		7.14 ± 0.80	
DP5102::mini-Tn*10* 1-1			0.042 ± 0.084
DP5102::mini-Tn*10* 1-2			0.039 ± 0.098
DP5102::mini-Tn*10* 3-2			0.123 ± 0.066
DP5102::mini-Tn*10* 4-1			−0.027 ± 0.021
DP5102::mini-Tn*10* 5-1			0.023 ± 0.049

[a] All strains were grown to stationary phase at 30°C unless indicated otherwise. Equal numbers of bacteria were diluted either 10- or 100-fold for the assay. Amount of Ail on the surface is based on the amount of secondary alkaline phosphatase-conjugated anti-mouse antibody that recognizes the Ail-specific monoclonal antibody. The amount of secondary antibody bound to intact cells was quantitated with a colorimetric assay for alkaline phosphatase activity.

level of the parental strain grown at 37°C (Table 3). The remaining mini-Tn*10* mutants entered CHO cells at a level similar to the level of the parental strain grown at 37°C. Chromosomal DNA from the mutants was probed with *inv*-specific, *ail*-specific, and transposon-specific probes. The *inv* and *ail* loci in the mutants were unaffected by the insertions (data not shown). All of the insertions appeared to be at the different sites in the mutants (data not shown).

***ail* gene expression in mutant DP5102::mini-Tn*10* 2c5.** The level of surface-localized Ail protein in the mutant DP5102::mini-Tn*10* 2c5 (containing plasmid pACYC184-km) was measured by whole cell ELISA analysis using an Ail-specific monoclonal antibody. This analysis suggested that there was over 200 times more Ail protein in the mutant than in the wild-type parent, at least as available on the surface to the monoclonal antibody (Table 4). To determine if this apparent increase in cell surface localization was due to either an increase in the level of transcription of the *ail* gene or an increase in the level of translation of *ail*-homologous mRNA, Northern (RNA) blot and Western blot (immunoblot) analyses were performed. In neither case was the level higher in the mutant than in its parent strain (data not shown). These results suggested that the mutation was not altering the total level of Ail in the cell but rather altering just that which is accessible to the monoclonal antibody and the tissue culture cell surface.

Serum resistance of mutant DP5102::mini-Tn*10* 2c5. Since the increase seen in entry by the mutants into CHO cells is due to an increase in Ail protein on the cell surface, it might be expected that other Ail-mediated properties might also be affected in these strains. The mini-Tn*10* insertion mutant DP5102::mini-Tn*10* 2c5 (containing plasmid pACYC184-km) was grown to stationary phase at 30°C and tested for resistance to complement-mediated killing. This mutant was slightly more sensitive to serum killing (data not shown). These results suggested that the mutation affecting Ail levels in the cell was somehow specific to the entry phenotype encoded by this protein.

Effect of an *ail* mutation on entry of DP5102::mini-Tn*10* 2c5 into CHO cells. An alternative explanation for why the mutation in DP5102::mini-Tn*10* 2c5 did not result in an increase in serum resistance in this strain over its parent is that the increased cell entry is not due to the change in amount of Ail on the cell surface but rather is due to an alteration in the levels or activity of another, heretofore unidentified invasin of *Y. enterocolitica*. To determine whether the increase in entry by DP5102::mini-Tn*10* 2c5 is indeed Ail mediated, an *ail* mutation was introduced into the mutant strain by gene replacement techniques as described in Materials and Methods. The *ail* mutant derivative of DP5102::mini-Tn*10* 2c5 is unable to enter CHO cells (Table 5), whether grown at 30 or 37°C, suggesting that the increased entry by DP5102::mini-Tn*10* 2c5 is indeed Ail mediated and not due to another *Y. enterocolitica* invasion gene product.

Characterization of mutant LPS. The fact that the total level of Ail protein in the cell was unaffected in DP5102::mini-Tn*10* 2c5 but that the level accessible to the monoclonal antibody was greatly increased suggested there might be some change in the surface of the mutant strain. Furthermore, the slightly

TABLE 5. Entry of an *ail* mutant of DP5102::mini-Tn*10* 2c5 into CHO cells

Strain	Entry efficiency[a]	
	Grown at 30°C[b]	Grown at 37°C
8081c	37.7	51.2
DP5102::mini-Tn*10* 2c5-52 (*ail*)	0.105	0.009
DP5102::mini-Tn*10* 2c5-53 (*ail*$^+$)	1.60	39.7
HB101	0.067	0.132

[a] Determined as described for Table 2.

[b] Growth temperature of DP5102::mini-Tn*10* 2c5 derivatives. In both experiments, 8081c was grown at 30°C and HB101 was grown at 37°C.

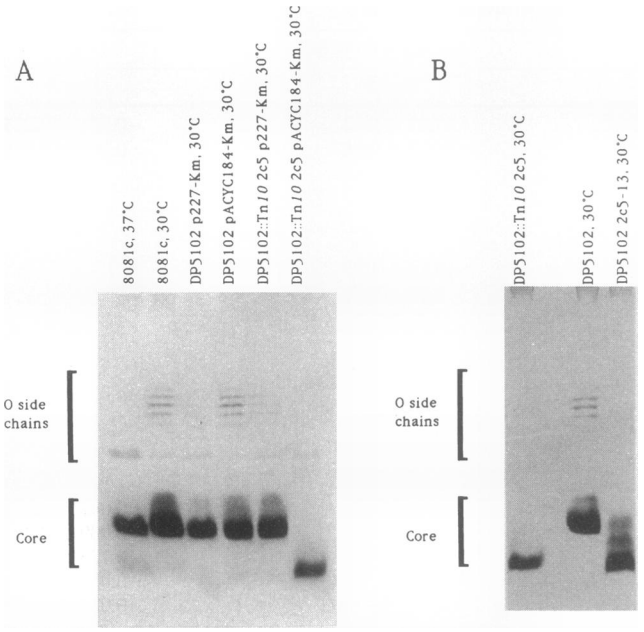

FIG. 1. LPS profile of DP5102::mini-Tn*10* 2c5. SDS-PAGE was performed on extracts of strains followed by silver staining. Samples in each lane are as indicated. The positions of the core and O side chains are shown.

higher serum sensitivity of this strain suggested that the mutation might be affecting LPS, which has been shown previously to have a role in serum resistance in several of the *Enterobacteriaceae*. The LPS profile of the mutant was examined by silver staining of polyacrylamide gels of mutant extracts. This analysis (Fig. 1A) demonstrates that the mutation in DP5102::mini-Tn*10* 2c5 affects the LPS in the cell such that there are no O side chains and the core is truncated. The lack of O side chains is most likely due to a mutation in core biosynthetic genes resulting in abnormal core to which O side chains cannot be added.

Transcomplementation of the DP5102::mini-Tn*10* 2c5 mutation. The wild-type copy of the gene mutated in DP5102::mini-Tn*10* 2c5 was isolated by first isolating the mutated copy, selecting for clones resistant to tetracycline, the marker carried by mini-Tn*10*. The cloned mutated gene was then used to probe a library of clones from the wild-type strain 8081c. A single clone, called p227, containing a 10.8-kbp insert of DNA into the vector pACYC184 was isolated (Fig. 2). The *apt* gene from pUC-4K was introduced into the vector portion of this recombinant molecule, making it possible to introduce it into DP5102::mini-Tn*10* 2c5. DP5102::mini-Tn*10* 2c5 containing the recombinant plasmid was then tested to determine if the mutation was complemented by the cloned DNA. All phenotypes tested, including cell entry (Table 3), surface Ail levels, as determined by ELISA analysis (Table 4), and LPS profile (Fig. 1A) were complemented by the recombinant molecule, confirming that this molecule contains the wild-type copy of the gene identified by the mutation in strain DP5102::mini-Tn*10* 2c5. The position of the complementing DNA on the cloned fragment was narrowed down from 10.8 kbp to 6.4 kbp by deletions and Tn*5* mutagenesis (Fig. 2). Tn*5* mutagenesis indicates that insertions all along the 6.4-kbp fragment destroy complementation of the mutation in DP5102::mini-Tn*10* 2c5. This information suggests that the mutation identified may be in one gene of an operon, affecting the activity not only of this gene but also of genes downstream.

Characterization of a mutant with an insertion directed to the locus cloned in p227. To confirm that the altered phenotypes in DP5102::mini-Tn*10* 2c5 are due to the mini-Tn*10* insertion and not due to a secondary mutation elsewhere in the strain, the cloned mini-Tn*10* insertion was crossed back into DP5102. A mutant derivative of DP5102, called DP5102 2c5-13, with an insertion directed to the locus cloned in p227 was constructed as described in Materials and Methods. Strain DP5102 2c5-13 was examined for LPS phenotype, entry into CHO cells, and surface-exposed Ail levels. The LPS profile of DP5102 2c5-13 was similar to that seen for DP5102::mini-Tn*10* 2c5 (Fig. 1B). In a cell entry assay, 3.04% ± 0.47% of the input DP5102 2c5-13 entered CHO cells, compared with 3.19% ± 0.76% of input DP5102::mini-Tn*10* 2c5. Cell surface levels of Ail protein in the defined mutant DP5102 2c5-13, as determined by whole cell ELISA, were similar to those of DP5102::mini-Tn*10* 2c5 (3.06 ± 0.905 and 5.44 ± 0.628, respectively). The similarity in all phenotypes in the defined mutant and mutant DP5102::mini-Tn*10* 2c5 demonstrates that it is the

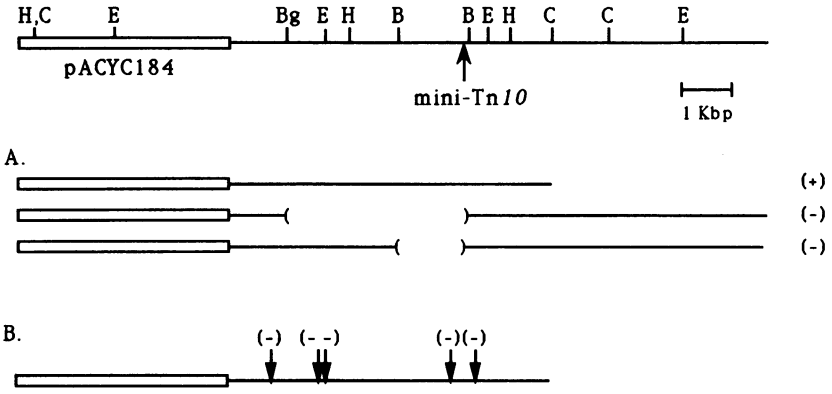

FIG. 2. Restriction map of clone p227. The position of the mini-Tn*10* insertion in mutant DP5102::mini-Tn*10* 2c5 is indicated. Restriction enzyme abbreviations: H, *Hin*dIII; C, *Cla*I; E, *Eco*RI; Bg, *Bgl*II; B, *Bam*HI. (A) Deletion analysis of clone p227. + and − indicate whether the clone complements both invasion phenotype and surface Ail levels. (B) Position of Tn*5* insertions in p227 containing a *Cla*I deletion. − indicates that insertion mutants do not affect either the invasion phenotype or surface Ail levels of DP5102::mini-Tn*10* 2c5 containing the different Tn*5* mutants. ▭, pACYC184 sequence; ——, *Y. enterocolitica* chromosomal DNA sequence.

mini-Tn*10* insertion that is responsible for the observed phenotype changes.

Characterization of Ail levels in other suppressors of the inability of DP5102 to enter mammalian cells. To determine if the increase in invasion of CHO cells by the other mini-Tn*10* and the Tn*5B50* mutants of DP5102 grown at 30°C was also due to an apparent increase in the level of Ail on the surface of the bacteria, whole cell ELISA analysis was performed with an anti-Ail monoclonal antibody. In all of the Tn*5B50* mutants, the level of Ail accessible to the antibody was over 20 times higher than the level in the parental strain (Table 4). This level is similar to those seen in DP5102::mini-Tn*10* 2c5. The level of surface-exposed Ail protein in the other mini-Tn*10* mutants was not increased to such a high degree, however. Instead, these mutants had levels lower than or similar to that of the wild-type strain grown at 37°C.

Characterization of the LPS profiles in other suppressors of DP5102 and complementation by clone p227. The fact that the Tn*5B50* mutants were similar to DP5102::mini-Tn*10* 2c5 both in levels of cell entry and in levels of surface-exposed Ail suggested that the mutations in all of these strains may be affecting the same property in the cell, that is, LPS. To determine if these mutants were also altered in their LPS, the LPS profiles were examined by PAGE (Fig. 3). All of these new mutants had altered LPS compared with the wild type. None had O side chains, and all but one had shortened core molecules. The fact that one mutant, DP5102::Tn*5B50* 3-2, had normal core indicates that it is the lack of a normal O side chain, not the alteration in core, that results in the increase in Ail-mediated cell entry by these mutants. To determine if any of these mutations mapped to the same locus as that mutated in 2c5, the cloned wild-type gene was introduced into the mutant strains and their LPS profiles were examined. Three of the mutants, DP5102::Tn*5B50* 1-2, 4-2, and 4-3, were complemented by this recombinant plasmid, indicating that the mutations do lie in the same locus as that in DP5102::mini-Tn*10* 2c5. The other seven were not complemented, suggesting that they lie in other genes required for synthesis of proper-length LPS. These other genes could include both structural and regulatory genes.

Analysis of *ail* gene expression in the remaining DP5102::mini-Tn*10* mutants. The levels of Ail protein on the surface of the five remaining mini-Tn*10* mutants was lower than (DP5102::mini-Tn*10* 1-1, 1-2, 4-1, and 5-1) or similar to (DP5102::mini-Tn*10* 3-2) the levels found in the wild-type parent grown at 37°C (Table 4). The LPS profile of these mutants was identical to that of the wild-type parent grown at 30°C, indicating that the mutation had not affected LPS biosynthetic genes (data not shown). To determine if the increase in surface-localized Ail protein was due to an increase in Ail levels as a result of increased transcription of the *ail* gene, the levels of *ail* mRNA were examined (Fig. 4). In all five mutants grown at 30°C, *ail* mRNA levels were higher than those in DP5102 grown at 30°C. In addition, in mutant DP5102::mini-Tn*10* 3-2 grown at 30°C, *ail* mRNA levels were higher than those in its wild-type parent grown at 37°C. In the remaining mutants, the levels were similar to those in the 37°C-grown wild-type parent. Thus, the increase in Ail protein on the surface of these mutants appears to be due to an increase in *ail* gene expression.

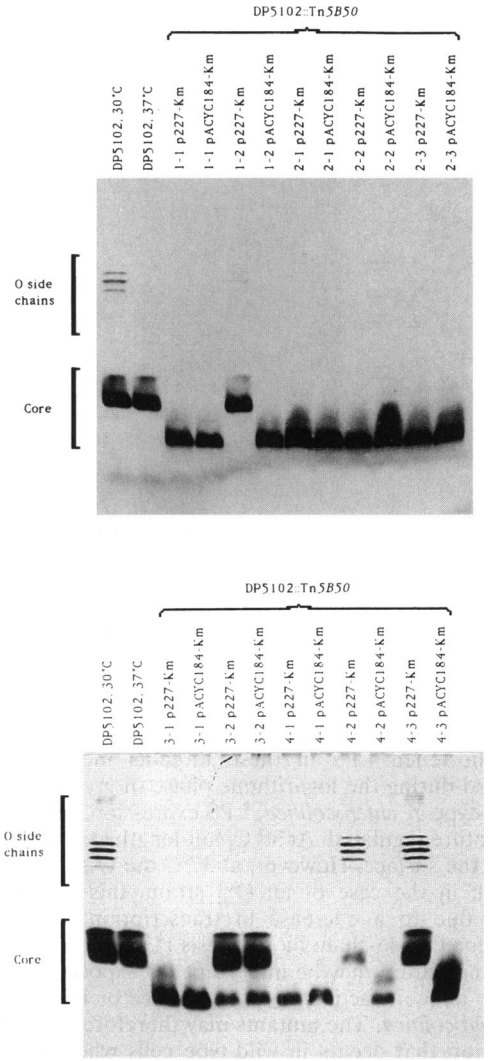

FIG. 3. LPS profiles of DP5102::Tn*5B50* mutants. Extracts from DP5102::Tn*5B50* mutants containing either p227-km or pACYC 184-km grown at 30°C were subjected to electrophoresis. Gels were treated with periodate and silver stained. The positions of the core and O-side-chain portions of the LPS molecules are indicated.

DISCUSSION

To examine the role of the *inv* gene in the ability of *Y. enterocolitica* to enter mammalian cells, an *inv* mutant derivative of *Y. enterocolitica* isolate 8081c was constructed. When grown at 30°C, this isolate does not enter cells. When the *inv* mutant strain is grown at 37°C, however, it entered CHO cells at a low level. Since the *ail* gene is expressed well at 37°C but not at 30°C, this low-level entry is likely Ail mediated. This hypothesis is supported by the fact that an *inv ail* double mutant grown at 37°C does not enter CHO cells.

To identify the genes whose products regulate Ail levels in the cell, the *inv* mutant strain was mutagenized with mini-Tn*10* or Tn*5B50* and mutants that would enter cells when grown at 30°C were isolated. Sixteen mutants, all of which enter cells better than their parent, were identified. Five of the mutants have increased expression of the *ail* gene. In the remaining 11, the accessibility of Ail protein to monoclonal antibodies, and presumably also to the mammalian cell surface, is increased. Analysis of the latter mutants indicates that the LPS is altered such that at 30°C there are no O side chains made and in all but one, the core is also altered. Since no *ail* gene expression can be detected in stationary phase at 30°C in any of these 11 mutants or their parental strain, the Ail protein that is exposed

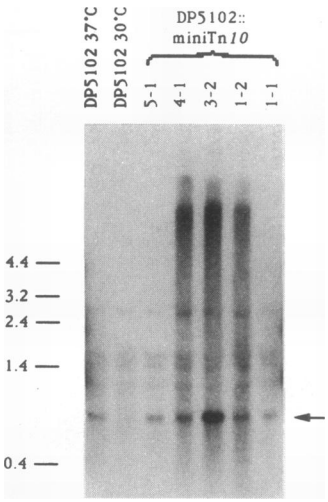

FIG. 4. *ail* gene expression in DP5102::mini-Tn*10* mutants. A 7.9-μg aliquot of total RNA from DP5102::mini-Tn*10* mutants grown to stationary phase at 30°C was electrophoresed on a formaldehyde-agarose gel and probed with a 0.4-kbp *Cla*I-*Acc*I fragment of DNA internal to the *ail* protein-coding region. The arrow indicates the position of the *ail* gene transcript. Sizes (in kilobases) of DNA markers are indicated at the left.

by the shortened LPS in these mutants must have been synthesized during the logarithmic phase of growth.

In wild-type *Y. enterocolitica*, LPS expression on the surface is temperature regulated. At 30°C, full-length O side chains are found on the surface. However, at 37°C the O side chains are shortened; in the case of an O:3 strain, this shortening of chains is due to a decrease in transcription of the genes required for O-side-chain biosynthesis (1). Shortening of LPS at this temperature may be important for exposure of the Ail protein to allow its action as an adherence or invasion factor for *Y. enterocolitica*. The mutants may therefore be mimicking the situation that occurs in wild-type cells when Ail protein function is required.

LPS, particularly the O-antigen moiety, has been shown in a number of gram-negative bacteria to have a role in resistance to serum killing (20, 25, 28). In the host, at 37°C, *Y. enterocolitica* LPS would not contribute to serum resistance since O antigen is shortened. Production of Ail and of YadA, a virulence plasmid-encoded protein that also has a role in serum resistance at 37°C (3), may therefore be required to allow this organism to avoid killing by host serum.

In addition to affecting the serum resistance phenotype of a number of bacteria, the LPS makeup of the cell has also been shown to have an effect on the invasion phenotype of some of the *Enterobacteriaceae. Salmonella choleraesuis* strains containing mutations in either the O side chains or the LPS core are unable to invade or transcytose a monolayer of MDCK epithelial cells (9). Thus, in this organism, in contrast to what is shown here with *Y. enterocolitica*, a lack of normal LPS decreases the ability of bacteria to interact with the mammalian cell surface. Voorhis et al. (27) have shown that when the gene for *Yersinia pseudotuberculosis* invasin, the *inv* gene, is introduced into *Shigella flexneri*, the bacteria do not enter cells. Mutants of these recombinant strains that do not enter cells can be generated, however; these bacteria have O-side-chain mutations. Thus, the function of both of the cell entry factors identified in *Yersinia* spp. appears to be inhibited by LPS molecules with multiply repeating O-side-chain units. This inhibition may simply be due to the fact that the longer LPS molecules hide the cell entry factors. Alternatively, it may be that the entry factors must be in the right context, that is, with LPS with shorter O side chains, to be fully functional.

Five of six mini-Tn*10* mutants that enter better than the parent when grown under conditions in which the *ail* gene is not expressed were identified. These mutants do not have altered LPS profiles. In at least one of the mutants, the level of *ail* mRNA is increased to levels above those seen in the wild-type strain grown under conditions in which the *ail* gene is expressed. These results suggest that there is a repressor of *ail* gene expression in bacteria grown to stationary phase at 30°C which is inactivated by the mini-Tn*10* insertion in the mutant strains. The putative repressor gene(s) is being characterized further. It is not clear why these mutants can enter mammalian cells better than their parent, as they have full-length LPS, which analysis of the other class of mutants indicates shields access of Ail to the mammalian cell surface. It may be that in these mutants with increased *ail* gene expression, the levels of Ail are high enough to overcome the shielding effect, allowing bacterium-host cell interaction to occur.

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